IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: WONG et al

Serial No. 09/856,339

Filed: 18 May 2001

For: PROCESS FOR OXIDISING TERPENES

DECLARATION

I, Professor Luet Wong, do hereby declare and state as follows:

1. I am a British subject of the Department of Chemistry, Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, UK. I presently have the position of University Lecturer in Chemistry. I have been working in the field of enzyme mutagenesis since 1990. My publications in this field are shown in the attached annex.

2. I am one of the inventors named for US Patent Application No. 09/856,339. I understand that the US Patent Office Examiner has objected that the oxidation method described in the claims of this patent application is obvious from the disclosure of GB-A-2294692, GB-A -2306485, US-A-6117661 and US-A-6100074 (hereafter referred to as the "cited documents"). I have been asked to comment on the Examiner's objection.

3. The work described in US Patent Application No. 09/856,339 concerns oxidation of terpenes by particular mutant monooxygenase enzymes. The claims of the application which are currently under consideration concern oxidation of limonene, pinene or a sesquiterpene; or a substituted derivative thereof, with the proviso that the substituent is not a halogen or does not comprise an oxygen atom.

4. I believe that the skilled person reading the cited documents would be able to conclude very little about the ability of the mutant P450 enzyme to successfully oxidise compounds. In particular, I believe that it would be impossible for the skilled person to come to any conclusions regarding the ability of the mutant P450 enzyme to oxidise limonene, pinene or a sesquiterpene. I will discuss in detail below why the data in the cited documents cannot be used to determine the substrate range of the mutant P450 enzyme. In addition I will also discuss why the differences between the compounds listed in the cited documents and the substrates mentioned in the present claims are significant enough for it not to be possible to make a meaningful comparison between the two sets of molecules.

5. Firstly when interpreting data concerning P450 enzymes it is important to bear in mind certain facts. The oxidation reaction carried out by a P450 enzyme is done via a complex

multi-step mechanism. All of the steps must occur for a compound to be oxidised by the P450 enzyme. However, it is known that when particular compounds are added to P450 enzymes only certain steps of the reaction occur, and successful oxidation of the compounds is not achieved. In particular, it is possible for "uncoupling" to occur so that NADH consumption and change in spin-state of the P450 enzyme becomes "uncoupled" from product formation. This is appreciated in England et al (1998) FEBS Letters 424, 271-4 which states in the second full paragraph of the left hand column of page 272 that:

"However, since the monooxygenase activity of P450 enzymes is well known to undergo uncoupling side reactions, not all of the NADH consumed by the system was necessarily utilised for substrate oxidation. It was therefore important to analyse the NADH turnover reactions for the present of substrate oxidation products, and determine the quantities of products formed so that both the rate of product formation and consequently the coupling efficiency could be calculated."

This is also appreciated in Atkins and Sligar (1988) J. Biol. Chem. 263, 18842-9 which discusses towards the bottom of the left hand column of page 18846 how the rates of NADH consumption "do not necessarily reflect rates of hydroxylated product formation since various substrate-cytochrome P450 complexes "uncouple" to produce differing amounts of hydrogen peroxide and water resulting from internal branch points within the cytochrome P450 reaction cycle".

Towards the bottom of the right hand column of page 18848 of Atkins and Sligar, it is noted:

"It is interesting, however, that camphane, which elicits a 46% high spin population, results in a drastic reduction in monooxygenase activity such that only 8% of the NADH consumed is utilised for hydroxylation."

Therefore neither change in spin state nor rate of NADH consumption can be used to determine successful oxidation of a compound by a P450 enzyme. Instead detection of the oxidised product is required to determine whether a compound is oxidised by a P450 enzyme. Thus the spin data or NADH consumption data in the cited documents cannot be used to infer that successful oxidation is occurring.

6. Similarly, the DTT data which is provided in the cited documents is a measure of the displacement by a test compound of DTT which is bound to the P450 enzyme (see third paragraph of page 12 of GB-A-2294692). The mere binding of a compound to a P450 enzyme cannot be used to determine whether or not oxidation of the compound occurs, as again an uncoupled reaction may occur instead.

7. Thus much of the data shown in the cited documents cannot be used to draw any conclusions regarding the substrate range of the mutant P450 enzyme.

8. Further, even if one made the assumption that the spin change data, NADH consumption data and DTT data shown in the cited documents was an indication of successful oxidation, the substrates mentioned in the present claims are significantly different from the compounds

disclosed in the cited documents. The compounds disclosed in the cited documents come within one or more of the following descriptions:
- simple alkyl compounds,
- aromatic compounds, or
- compounds activated by the presence of an oxygen or halogen.

None of the substrates mentioned in the claims fall within the above descriptions. Oxidation data for simple alkyl compounds cannot be used to predict whether the substrates mentioned in the present claims (i.e. a limonene, pinene or a sesquiterpene) could be oxidised by a mutant P450 enzyme. As can be seen from Tables 1 and 2 on pages 41 and 42 of the present patent application the substrates mentioned in the claims are large molecules which are significantly more complex than the alkanes listed in Tables 2(a) and 2(g) of the cited documents. Enzymes are known to be highly specific in regard to the compounds they will accept as substrates. Therefore even if one accepts that the alkyl compounds listed in Tables 2(a) and 2(g) are oxidised by the mutant P450 enzyme this cannot be used to infer that the enzyme will also oxidise the substrates mentioned in the present claims.

Aromatic compounds have very different molecular and electronic structures from the substrates mentioned in the present claims, and therefore oxidation data concerning aromatic compounds cannot be used to predict whether or not limone, pinene or a sesquiterpene would be oxidised by a mutant P450 enzyme.

Compounds activated by an oxygen or halogen atom are of course excluded from the present claims. Therefore the data concerning these compounds which are shown in the cited documents is not relevant.

9. The only compounds in the cited documents which have some similarity to the substrates mentioned in the claims are carvone in Table 2(a) and camphorquinone, fenchone and dicylopentadiene in Table 2(h). The first three of these compounds have activating oxygen atoms (note the structure shown for carvone is incorrect as the oxygen is not shown).

Further, for these four compounds the spin state data and DTT data provided in the cited documents often shows little difference between the wild-type and mutant P450 enzymes. Thus making it difficult to interpret whether or not oxidation is occurring.

10. Given the above differences between the compounds listed in the cited documents and the substrates mentioned in the presents claims, the data shown in the cited documents cannot be used to predict whether or not the substrates mentioned in the present claims can be oxidised by a mutant P450 enzyme.

11. In conclusion, the spin change data, NADH consumption data and DTT data shown in the cited documents cannot be used to infer that oxidation is occurring. Further the compounds listed in the cited documents are on the whole so different in structure from the substrates mentioned in the present claims that the data in the cited documents cannot be used to predict whether or not the substrates mentioned in the present claims will be oxidised by a mutant P450 enzyme.

12. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of this declaration, the patent application, or any patents issuing thereon.

Signed
Professor Luet Wong Luet Wong

This 18 Day of August 2004.